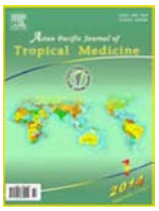
Asian Pac J Trop Med 2014; 7(Suppl 1): S204-S211

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60233-9

Effect of certain natural products and organic solvents on quorum sensing in *Chromobacterium violaceum*

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ARTICLE INFO

Article history:
Received 2 Jun 2014
Received in revised form 5 Jun 2014
Accepted 15 Jun 2014
Available online 19 Aug 2014

Keywords:
Quorum sensing
Microwave assisted extraction (MAE)
Prodigiosin
Violacein
HPLC
Streptomycin
Solvent
Antipathogenic

ABSTRACT

Objective: To investigate the effect of seed extracts of *Pongamia pinnata*, *Pyrus pyrifolia*, and *Manilkara hexandra*, bacterial pigment prodigiosin, and three organic solvents (ethanol, methanol, and dimethylsulfoxide), on quorum sensing (QS) in *Chromobacterium violaceum* (*C. violaceum*).

Methods: *C. violaceum* was challenged with plant extracts prepared by microwave assisted extraction method, prodigiosin, and organic solvents. Effect of these test substances on *C. violaceum* growth, and quorum sensing regulated pigment (violacein) production was studied by broth dilution assay. High performance liquid chromatography was also applied to generate chromatographic fingerprint of the active extracts. Effect of sub-minimum inhibitory concentration level of the antibiotic streptomycin on quorum sensing regulated pigment production was also studied.

Results: *Pongamia pinnata* seed extracts and prodigiosin were found to possess anti-QS, and *Manilkara hexandra* and *Pyrus pyrifolia* seed extracts to possess QS-enhancing effect in *C. violaceum*. Dimethylsulfoxide was found to enhance violacein production, whereas ethanol and methanol reduced violacein production in *C. violaceum*. Streptomycin at sub-minimum inhibitory concentration level was able to significantly arrest QS-regulated pigment production in *C. violaceum* and *Serratia marcescens*.

Conclusions: Prodigiosin and the seed extracts used in this study could affect quorum sensing in *C. violaceum* to a notable extent. Results of this study also emphasize the importance of inclusion of appropriate solvent controls (negative controls) in bioassays designed for screening of antimicrobial and/or anti-QS compounds. Antipathogenic potential of low concentrations of streptomycin was also demonstrated.

1. Introduction

Either for validation of traditional use or for the purpose of drug discovery, major focus has been on the antimicrobial potential of the natural products including plant extracts; wherein an assay is designed to investigate whether a natural product can kill or inhibit the test pathogen[1]. Enough attention has not been paid to the antipathogenic potential of natural products. Inhibition of quorum sensing (QS) by natural products is a good example of their antipathogenic effect. Preparations capable of reducing or inhibiting QS in bacteria may devoid the bacteria of their virulence, partially or fully, without necessarily killing them. QS is a means of cell-to-cell communication among bacteria, whereby they synthesize, exchange, and sense small signal molecules[2], *e.g.* N-acyl-homoserine lactones (AHLs) produced by Gram-negative bacteria. QS signalling systems of pathogens being central regulators of the expression of virulence factors, are viewed as attractive targets for the development of new therapeutics[3]. Few QS inhibitors such as furanone have been reported with the potential of being used in treatment of infection[4,5].

A variety of density-dependent multicellular behaviour such as pigment production, antibiotic production, and biofilm formation have been reported in pathogenic bacteria. Synthesis of the violet pigment violacein by the Gram-negative bacterium *Chromobacterium violaceum* (*C. violaceum*) occurs in a QS dependent fashion. *C. violaceum*

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Foundation Project: Supported by Nirma Education and Research Foundation.

has been used as model bacterium while screening natural products for anti-QS property[6]. Any alteration in the pigment producing ability of *C. violaceum* under the influence of test compound(s) can easily be quantified photometrically.

The present study aimed at investigating the effect of seed extracts of three different plants *viz.* *Manilkara hexandra* Roxb (Sapotaceae) (*M. hexandra*), *Pongamia pinnata* Lam (Papilionaceae) (*P. pinnata*), and *Pyrus pyrifolia* Burm (Rosaceae) (*P. pyrifolia*), on bacterial QS. Common Indian names for these plants are rayan, karanj, and nashpati respectively. Mahida and Mohan reported *M. hexandra* (leaves or tubers) extracts to possess antibacterial activity[7]. Chanda and Parekh reported antimicrobial activity of *M. hexandra* leaf extracts against few Gram-positive and Gram-negative bacteria, as well as fungi[8]. *P. pinnata* seed oil has been reported for antibacterial as well as antifungal property[9]. *P. pinnata* bark, leaves, and seed extracts were reported for their antioxidant and antimicrobial properties by Sajid *et al*[10]. Seeds of *P. pinnata* are known to be antileprotic, and are prescribed in bronchitis and whooping cough. The aqueous extract of seeds is known to possess antiviral activity against herpes simplex viruses[11].

Additionally, anti-QS potential of the bacterial pigment prodigiosin was also investigated. Prodigiosin is a red linear tripyrole pigment produced by some strains of *Serratia*. It is a secondary metabolite of potential clinical interest, possessing antimicrobial, immunosuppressive, and anti-cancer properties[12]. In this process, we also studied effect of three solvents [ethanol, methanol and dimethylsulfoxide (DMSO)] on violacein production in *C. violaceum*, as these solvents are commonly used for extraction or for reconstitution of the dry extracts.

2. Materials and methods

2.1. Test organisms

C. violaceum (MTCC 2656), and *Serratia marcescens* (MTCC 97) (*S. marcescens*) were procured from Microbial Type Culture Collection, Chandigarh, India.

2.2. Plant material

Seeds of *P. pinnata* were collected from its tree in the Nirma University campus. Seeds of *M. hexandra* and *P. pyrifolia* were procured during October 2013 to February 2014, from the fruits purchased from local market in the city of Ahmedabad. They were authenticated for their unambiguous identity by Dr. Himnashu Pandya, Department of Botany, Gujarat University, Ahmedabad.

2.3. Extraction of plant material

Seeds were extracted in three different solvents (Merck, Mumbai, India)-acetone, methanol, and ethanol (50%) by microwave assisted extraction method. One gram of dry seed powder was soaked into 50 mL of solvent, and subjected to microwave heating (Electrolux EM30EC90SS) at 720 W. Total heating time was kept 120, 90 and 70 seconds for acetone, methanol, and ethanol, respectively, with intermittent cooling. This was followed by centrifugation (at 7500 r/min for 20 min), and filtration with Whatman No. 1 paper (Whatman International Ltd., Maidstone, England). Solvent was evaporated from the filtered extract and then the dried extracts were reconstituted in DMSO for bioassay. Reconstituted extracts were stored under refrigeration for further use. Extraction efficiency was calculated as percentage weight of the starting dried plant material.

2.4. Prodigiosin extraction

S. marcescens was grown in nutrient broth for 60 h at 28 °C under shaking condition (at 200 r/min). After quantifying growth at 625 nm, prodigiosin extraction was carried out from *S. marcescens* culture as described in Vaikunthvasan *et al*[13]. Briefly, 100 mL of the culture broth was centrifuged (Nüve NF 800 R) at 7500 r/min for 15 min. Centrifugation was carried out at 4 °C, as prodigiosin is a temperature-sensitive compound (Williams, 1973)[14]. The resulting supernatant was discarded. Remaining cell pellet was resuspended in 100 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 7500 r/min for 15 min at 4 °C. Prodigiosin in the resulting supernatant was estimated by measuring OD at 535 nm[15]. Spectrum of the extracted prodigiosin was also generated in the wavelength range 400-700 nm, wherein purity of the preparation was confirmed by observation of a single peak (data not shown). After evaporating methanol, remaining prodigiosin content was reconstituted in DMSO. Concentration of the prodigiosin was calculated using molar extinction coefficient of prodigiosin at 535 nm *i.e.* 1.12×10^5[16]. Concentration of the prodigiosin stock prepared by us was found to be 3.88 mg/mL. This was then tested at 1%-3% v/v corresponding to 38.80-116.40 μg/mL concentration.

2.5. QS assay

C. violaceum was challenged with different concentrations of all the three seed extracts, and prodigiosin. Nutrient broth (HiMedia, Mumbai) was used as growth medium. All the extracts of *P. pyrifolia* were getting precipitated in nutrient broth, and hence could not be tested (except the methanolic extract, where precipitation was observed only at higher concentrations). Inoculum density of the test organisms was adjusted to that of 0.5 McFarland standard. Test extract followed by inoculum was added into nutrient broth tubes. Extracts or proidigiosin (reconstituted in DMSO) were serially diluted into respective tubes. A DMSO control was included in all assays[17]. Gentamicin and streptomycin (HiMedia) served as positive control. Appropriate abiotic controls (containing media and extract, but no inoculum) were also set. Tubes were incubated at 35 °C for 18-20 h, before being read at 660 nm (Elico SL210 spectrophotometer) for estimation of growth.

Following estimation of growth, the tubes were subjected

to violacein extraction as described by Choo *et al*[6]. Briefly, 2 mL of the culture broth was centrifuged (Eppendorf 5417 R) at 10000 r/min for 15 min, and the resulting supernatant was discarded. The remaining cell pellet was resuspended into 2 mL of DMSO (Merck, Mumbai) , and vortexed, followed by centrifugation at 10000 r/min for 15 min. The violacein extracted in the supernatant was estimated by measuring OD at 585 nm[18]. Violacein unit was calculated as the ratio-(OD_{585}/OD_{660}) which gives an indication of violacein production per unit of growth.

Effect of organic solvents (ethanol, methanol, and DMSO) on QS-regulated violacein production was also studied using the method described above.

2.6. High performance liquid chromatography (HPLC)

Extracts dissolved in their respective solvent (methanol/ ethanol/ acetone) were filtered through a polyvinylidene fluoride hydrophilic membrane syringe filter (0.22 μm, Himedia) and 10 μL aliquots of the filtrate were injected into HPLC system (Agilent 1260) with a ZORBAX Eclipse Plus C_{18} column (4.6 mm×250 mm). Mobile phase consisted of orthophosphoric acid (0.05%): acetonitrile (Merck). A gradient elution was set at 1 mL/min. A gradient of mobile phase was applied by varying the relative proportion of both component solvents of the mobile phase. Ratio employed was solvent A to solvent B (0-6 min: 5% B, 6-15 min: 15% B, 15-35 min: 20% B, 35-40 min: 40% B; A: orthophosphoric acid, B: acetonitrile). Detection was carried out at 220/270 nm.

2.7. Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean±SD. Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

3. Results

Table 1 lists extraction efficiency and reconstitution efficiency of different extracts. Results of experiments with plant extracts and prodigiosin are presented in Table 2.

Table 1
Extraction and reconstitution efficiency for all the seed extracts.

Seed	Solvent	Extraction efficiency (%)	Reconstitution efficiency (%)
P. pinnata	Ethanol (50%)	21.58	19.28
	Methanol	17.08	29.86
	Acetone	17.42	26.41
M. hexandra	Ethanol (50%)	13.24	83.23
	Methanol	13.78	74.31
	Acetone	17.92	5.80
P. pyrifolia	Ethanol (50%)	10.40	78.85
	Methanol	9.78	77.09
	Acetone	9.32	17.59

All the three extracts of *P. pinnata* exhibited anti-QS activity, as evident from reduced violacein production in presence of these extracts. Maximum inhibition (82.92%) of violacein production was achieved, when *C. violaceum* was challenged with ethanolic extract of *P. pinnata* at 1000 μg/mL (Figure 1). Acetone extract of *P. pinnata* proved inferior with respect to QS inhibition potential, to remaining two extracts of the same seed. The antimicrobial effect (*i.e.* inhibition of growth) of *P. pinnata* extract was lesser than the anti-QS effect, indicating that these extracts were capable of inhibiting QS-regulated violacein production without killing the bacterial cells to any great extent. Interestingly, methanolic extract of *P. pinnata* enhanced QS at 250 μg/mL without affecting the growth significantly, and the same extract had an anti-QS activity in the concentration range 500-1000 μg/mL, with concurrent inhibition of growth. Acetone extract of *P. pinnata* showed the maximum anti-QS activity at 500 μg/mL. This extract exhibited neither significant antimicrobial nor anti-QS effect at any of the other concentrations tested.

Figure 1. Anti-QS potential of ethanolic extract of *P. pinnata*.
Image of the cell-free supernatant containing extracted violacein. GC: Growth control; NC: Negative control; EC1: 250 μg/mL concentration of the test extract; EC2: 500 μg/mL concentration of the test extract; EC3: 750 μg/mL concentration of the test extract; EC4: 1000 μg/mL concentration of the test extract. Lesser or no violet colour typical of violacein can be seen in experimental tubes as compared to the negative control (which contained DMSO but no extract) tube. Enhanced violacein production in negative control compared to growth control (with no DMSO) is also visible.

None of the extracts of *M. hexandra* could inhibit QS in *C. violaceum*. Its acetone extract could inhibit the growth of *C. violaceum* upto certain extent, but had no significant effect on violacein production; whereas ethanolic and methanolic extracts of this seed enhanced violacein production with simultaneous promotion of growth. An increment of 344.44% in violacein unit was observed, when *C. violaceum* was incubated with ethanolic extract of *M. hexandra* (500 μg/mL), which explains the observed heavy increase (438.46%) in violacein production despite not so heavy increase (32.82%) in growth.

Methanolic extract of *P. pyrifolia* was able to enhance QS-regulated violacein production in *C. violaceum* with simultaneous decrease in growth of the organism. Under influence of this extract organism produced more violacein from a lesser cell density, suggesting an increase in per cell violacein production. For example, this extract at 1000 μg/mL could enhance the ratio of violacein to growth (OD_{585}/OD_{660}) by 175.67%. Thus methanolic extract of *P. pyrifolia* proved antimicrobial but not anti-QS.

Table 2
Effect of seed extracts and prodigiosin on QS in *C. violaceum*.

Test sample		Concentration (μg/mL)	Growth (OD_{660}) (mean±SD)		% Change compared to control	Violacein (OD_{585}) (mean±SD)		% Change compared to control	Volacein unit (OD_{585}/OD_{660})		%Change compared to control
			C	E		C	E		C	E	
P. pinnata	Anti-QS effect of ethanolic extract	250	1.73±0.01	1.15±0.01	−33.52[b]	0.67±0.05	0.30±0.00	−55.22[a]	0.38	0.26	−31.57
		500	1.57±0.08	1.04±0.01	−33.75[a]	0.41±0.02	0.12±0.00	−70.73[b]	0.26	0.11	−57.69[b]
		750	1.57±0.08	0.90±0.03	−42.67[b]	0.41±0.02	0.10±0.00	−75.60[b]	0.26	0.11	−57.69[b]
		1 000	1.57±0.08	0.93±0.00	−40.76[b]	0.41±0.02	0.07±0.00	−82.92[b]	0.26	0.07	−73.07[b]
	Anti-QS effect of methanolic extract	250	1.21±0.05	1.46±0.10	20.66	0.28±0.00	0.42±0.03	50.00[a]	0.23	0.28	21.73[a]
		500	1.21±0.05	0.93±0.00	−23.14[a]	0.28±0.00	0.17±0.00	−39.28[b]	0.23	0.18	−21.73[a]
		750	1.40±0.01	0.86±0.01	−38.57[b]	0.25±0.00	0.15±0.01	−40.00[b]	0.17	0.17	0.00
		1 000	1.56±0.01	0.83±0.03	−46.79[b]	0.46±0.00	0.11±0.00	−76.08[b]	0.29	0.13	−55.17[b]
	Anti-QS effect of acetone extract	250	1.48±0.11	1.28±0.01	−13.51	0.48±0.05	0.38±0.01	−20.83	0.32	0.29	−9.37
		500		1.31±0.01	−11.48		0.29±0.01	−39.58[a]		0.22	−31.25[a]
		750		1.45±0.06	−2.02		0.47±0.08	−2.08		0.32	0.00[a]
		1 000		1.37±0.05	−7.43		0.46±0.05	−4.16		0.33	3.12[a]
M. hexandra	QS enhancement by ethanolic extract	250	1.31±0.00	1.28±0.00	−2.2[a]	0.13±0.01	0.24±0.00	84.61[b]	0.09	0.18	100.00[b]
		500	1.31±0.00	1.74±0.00	32.82[b]	0.13±0.01	0.70±0.00	438.46[b]	0.09	0.40	344.44[b]
		750	1.31±0.00	1.80±0.02	37.40[b]	0.15±0.00	0.68±0.00	353.33[b]	0.11	0.37	236.36[b]
		1 000	1.27±0.00	1.89±0.10	48.81[a]	0.27±0.00	0.64±0.00	137.03[b]	0.21	0.33	57.14[b]
	QS enhancement by methanolic extract	250	1.20±0.02	1.22±0.02	1.66	0.36±0.03	0.31±0.01	−13.88	0.30	0.25	−16.66
		500	1.20±0.02	1.42±0.04	18.33[a]	0.36±0.03	0.42±0.02	16.66	0.30	0.29	−3.33
		750	1.25±0.01	1.90±0.07	52.00[b]	0.26±0.00	0.82±0.08	215.38[a]	0.20	0.43	115.00[b]
		1 000	1.25±0.01	1.94±0.05	55.20[b]	0.26±0.00	0.67±0.04	157.69[b]	0.20	0.34	70.00[b]
	Acetone extract	250	1.85±0.03	1.49±0.00	−19.45[b]	0.62±0.02	0.60±0.01	−3.22	0.33	0.40	21.21[a]
		500		1.62±0.03	−12.43[a]		0.55±0.02	−11.29	0.33	0.33	0.00
		750		1.56±0.01	−15.67[b]		0.58±0.02	−6.45	0.33	0.37	12.12[a]
		1 000		1.49±0.02	−19.45[b]		0.56±0.03	−9.67	0.33	0.37	12.12[b]
P. pyrifolia	QS enhancement by methanolic extract	250	1.28±0.02	1.34±0.03	4.68	0.18±0.00	0.21±0.01	16.66	0.14	0.15	7.14
		500	1.30±0.00	0.70±0.00	−46.15[b]	0.68±0.00	0.80±0.02	17.64[a]	0.52	1.14	119.23[b]
		750	1.09±0.00	0.49±0.00	−55.04[b]	0.89±0.00	1.03±0.00	15.73[b]	0.81	2.10	159.25[b]
		1 000	1.08±0.00	0.46±0.00	−57.40[b]	0.80±0.01	0.94±0.01	17.50[b]	0.74	2.04	175.67[b]
Prodigiosin	Anti-QS effect of prodigiosin	38.8	0.94±0.01	0.73±0.01	−22.34[b]	0.10±0.00	0.08±0.01	−20.00	0.10	0.10	0.00
		77.6	0.92±0.01	0.79±0.00	−14.13[b]	0.09±0.01	0.06±0.00	−33.33	0.09	0.07	−22.22
		116.4	0.89±0.01	0.79±0.00	−11.23[a]	0.11±0.00	0.06±0.00	−45.45[b]	0.12	0.07	−41.66

[a]$P<0.05$;[b]$P<0.01$, minus sign indicates a decrease over control; C: control; E: experimental

Prodigiosin till 77.6 μg/mL was having little antimicrobial effect, but no significant anti-QS effect. At 116.4 μg/mL it had little antimicrobial and considerable anti-QS effect.

The ethanolic extract of *P. pinnata* showing maximum anti-QS activity, and two of the *M. hexandra* extracts showing heavy QS-enhancing effect were subjected to HPLC (Figure 2). As crude extract may show considerable lot-to-lot variation, chromatography profile becomes useful in characterization of bioactive natural extracts. Chromatograms of individual bioactive extract can serve as chromatographic fingerprint for that particular extract[19], which may then be useful for standardization of crude extracts. Seeds and seed oil of *P. pinnata* are reported to contain karanjin, pongamol, pongapin, and kanjone. All parts of *M. hexandra* are reported to contain alpha-beta amyrin, cinnamates, alpha-sipnasterol, quercetin, and ursolic acid[11].

Effect of three different organic solvents (DMSO, methanol, and ethanol) on QS-regulated violacein production was also evaluated. DMSO was able to enhance violacein production in a dose-dependent manner, simultaneously exerting inhibitory effect on *C. violaceum* growth. Thus under the influence of DMSO violacein production by individual cell experienced an increase (Table 3). Methanol at 1% v/v affected neither growth nor violacein production significantly, at 2% v/v it inhibited the growth by nearly 12% without affecting violacein production. Methanol from 3%-5% v/v inhibited growth and violacein production in *C. violaceum* in a dose-dependent fashion. Ethanol was able to inhibit *C. violaceum* growth at all tested concentrations. It could also reduce violacein production significantly at 2%-5% v/v concentration. Despite a reduction of 13.04% in cell density at 1% v/v ethanol, no significant reduction in violacein production was observed. This may happen because perhaps the minimum cell density (*i.e.* quorum) required for violacein production at par with control was achieved despite minor growth inhibition in presence of 1% v/v ethanol. Same explanation can be offered for methanol at 2% v/v concentration. All the three test solvents inhibited growth of *C. violaceum* in a dose-dependent manner.

Streptomycin (HiMedia) was used as a positive control during all these experiments. Interestingly this antibiotic at high concentration exhibited antimicrobial, and at low concentration exhibited anti-QS activity (Table 4). This antibiotic had an minimum inhibitory concentration (MIC) of 2.5 μg/mL against *C. violaceum*. At sub-MIC level of 2 μg/mL, it inhibited growth by 32.44%, and interestingly, QS by 100%.

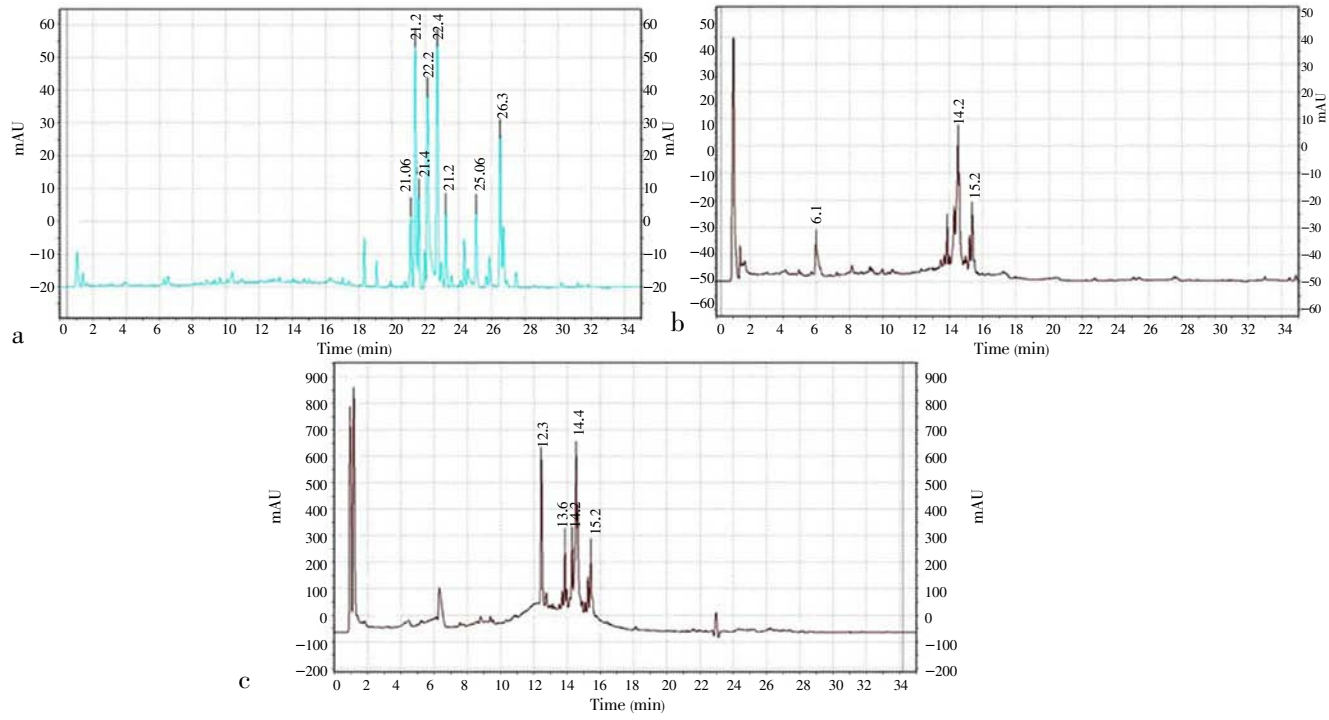

Figure 2. HPLC profile of active extracts with retention times (in min) marked on the peaks.
a: HPLC profile of ethanolic extract of *P. pinnata*; b: HPLC profile of methanolic extract of *M. hexandra*; c: HPLC profile of ethanolic extract of *M. hexandra*.

Table 3

Effect of different solvents on QS in *C. violaceum*.

Solvent	Concentration (% v/v)	Growth (OD_{660}) (mean±SD)		% Change compared to control	Violacein (OD_{585}) (mean±SD)		% Change compared to control	Volacein unit (OD_{585}/OD_{660})		% Change compared to control
		C	E		C	E		C	E	
DMSO	1	1.04±0.00	1.01±0.01	−2.88	0.16±0.00	0.19±0.00	18.75[a]	0.15	0.18	20.00
	2		0.95±0.01	−8.65[a]		0.20±0.00	25.00[b]	0.15	0.21	40.00
	3		0.87±0.01	−16.34[b]		0.22±0.00	37.50[b]	0.15	0.25	66.66[b]
	4		0.85±0.00	−18.26[b]		0.23±0.00	43.75[b]	0.15	0.27	80.00[a]
	5		0.86±0.01	−17.30[b]		0.26±0.00	62.50[b]	0.15	0.30	100.00[b]
Methanol	1	1.52±0.02	1.48±0.03	−2.63	0.36±0.00	0.35±0.00	−2.77	0.23	0.23	0.00
	2		1.34±0.02	−11.84[a]		0.35±0.00	−2.77	0.23	0.26	13.04
	3		1.17±0.03	−23.02[b]		0.29±0.01	−19.44[a]	0.23	0.24	4.34
	4		1.01±0.00	−33.55[b]		0.23±0.00	−36.11[b]	0.23	0.22	−4.34
	5		0.42±0.01	−72.36[b]		0.01±0.00	−97.22[b]	0.23	0.02	−91.30[b]
Ethanol	1	1.84±0.01	1.60±0.00	−13.04[b]	0.62±0.04	0.58±0.03	−6.45	0.33	0.36	9.09
	2		1.43±0.04	−22.28[b]		0.40±0.02	−35.48[a]	0.33	0.27	−18.18
	3		1.17±0.04	−36.41[b]		0.28±0.01	−54.83[a]	0.33	0.23	−30.30
	4		0.20±0.01	−89.13[b]		0.03±0.00	−95.16[b]	0.33	0.15	−54.54[a]
	5		0.02±0.00	−98.91[b]		0.01±0.00	−98.38[b]	0.33	0.50	51.55

[a]$P<0.05$; [b]$P<0.01$

Table 4

Effect of sub-MIC level of streptomycin on QS regulated pigment production.

Organism	Streptomycin (μg/mL)	Growth (OD_{625})			OD_{535} (for prodigiosin) OR OD_{585} (for violacein)		
		Control (mean±SD)	Experimental (mean±SD)	% Inhibition	Control (mean±SD)	Experimental (mean±SD)	% Reduction in pigment production
S. marcescens	5	0.624±0.003	0.599±0.001	5.47[C]	0.213±0.016	0.058±0.000	82.77[C]
C. violaceum	2	0.598±0.006	0.404±0.004	32.44[C]	0.100±0.010	0.000±0.000	100.00[C]

[C]$P<0.001$; above 2 μg/mL of streptomycin, *C. violaceum* was inhibited completely.

4. Discussion

Our study has identified *P. pinnata* extracts to possess appreciable anti-QS potential. These extracts could inhibit QS-regulated violacein production in *C. violaceum* at concentration ranging from 250-1 000 μg/mL. Prodigiosin (at 116.4 μg/mL) could reduce violacein production to almost half of that produced by control. Though it is difficult to draw direct comparison among various natural products reported to have anti-QS potential due to variations in- the methodology employed, microbial strains used, extraction method applied, *etc*; a broad comparison may not be out of place in having an idea about their relative efficacy. Vasavi *et al*. reported ethyl acetate fraction of *Syzygium cumini* and *Pimenta dioica* extracts could completely inhibit violacein production in the concentration range 0.75-1.00 mg/mL[20]. Clove oil at 0.2%-1.6% v/v could inhibit QS-regulated swarming motility in *Pseudomonas aeruginosa* (*P. aeruginosa*)[2]. Vanilla extract was shown to possess QS-inhibitory activity at 1%-2% w/v[6]. Plants such as carrot, garlic, pea seedlings, soybean, tomato, and water lily are known to produce compounds interfering with bacterial QS[3]. Halogenated furanones from the red alga *Delisea pulchra* are also known as anti-QS compounds[21]. Methanolic extract of *Capparis spinosa* demonstrated anti-QS and antibiofilm activity at 0.5-2.0 mg/mL[22].

Adonizio *et al*. reported anti-QS activity of six different southern Florida plants, wherein they found water extracts to be more effective[1]. *Vanilla planifolia* beans extracted using 75% v/v aqueous methanol were found to inhibit QS in *C. violaceum*[6]. In the present study, we have also found that polar solvents (water-ethanol mixture and methanol) proved good at extracting anti-QS compounds from *P. pinnata* seeds, and QS-enhancing compounds from *M. hexandra* seeds. Acetone could not extract as many QS-affecting compounds as either ethanol or methanol. Some studies avoid use of aqueous extracts while screening crude extract for *in vitro* antimicrobial property, as water-soluble compound are commonly more effective as inhibitors of pathogen adsorption making it difficult to identify them through the commonly used screening techniques[23]. In a study by Eloff (mentioned in[23]) for examining a variety of solvents for their ability to solubilize plant antimicrobials, acetone was given highest rating, and water received lowest ranking. It seems that the solvents good at extracting antimicrobial compounds may not be necessarily equally good at extracting anti-QS compounds.

In the present study three of the test extracts (ethanolic and methanolic extracts of *M. hexandra*, and methanolic extract of *P. pyrifolia*) were found to enhance QS-regulated violacein production in *C. violaceum*. Usually studies are designed to screen for anti-QS compounds rather than QS-enhancing compounds. However, phytocompounds which can enhance violacein production and/ or secretion in *C. violaceum* can also be of interest, as violacein is an important bioactive molecule with attractive potential therapeutic applications. This pigment is known to possess antibacterial, antifungal, antiviral, trypanocidal, anti-leishmanial, and antitumor activities[24-27]. Potential applications of violacein include its use as antibacterial agent in bandages, in preparation of antibacterial textiles, *etc*. Violacein has also been shown to work synergistically with many commercial antibiotics, and thus may be applied as drug in combination with other antimicrobials[27]. Many studies have been recorded in literature which were designed for achieving higher violacein production and/or extraction[28,29].

Though antimicrobial property of prodigiosin has been known[13], its anti-QS property has not received much attention. Prodigiosin was found to inhibit marine fouling bacteria like *Alteromonas* spp. and *Gallionella* spp. with MIC of 6.75 μg/mL and minimum bactericidal concentration of 12.5 μg/mL. Prodigiosin could also inhibit cyanobacteria on glass surface[30]. Prodigiosin analog have been reported to possess antifungal activity against *Trichophyton* spp[31]. Gram-negative bacteria such as *P. aeruginosa* and *Salmonella typhi* were reported for their susceptibility to prodigiosin by Sumathi *et al*[32]. They found multiple pathogenic bacterial strains to be inhibited by prodigiosin at 50-140 μg/mL, and different pathogenic fungi were inhibited by prodigiosin at approximately 80-450 μg/mL. Bacterial pigments having anti-QS activity can be expected to affect different QS-regulated attributes in bacteria such as biofilm formation, pigment production, virulence, *etc*. Prodigiosin has been shown to be capable of interacting with DNA, and inhibiting topoisomerases[33].

In addition to plant extracts and prodigiosin, we also evaluated the effect of three different organic solvents (DMSO, methanol and ethanol) on QS-regulated violacein production. All the three test solvents inhibited growth of *C. violaceum* in a dose-dependent manner. Growth inhibitory effect of these solvents against few bacteria was earlier reported by us in context of determining MIC of various antimicrobials[17]. Though effect of organic solvents commonly employed in bioassays on microbial growth has been investigated by few workers[34-37], their effect on QS in bacteria has not received much attention. As the present study has shown that solvents like DMSO, methanol, and ethanol can have considerable influence on growth as well as QS-regulated pigment production in *C. violaceum* (which is used as a model organism for QS studies), it becomes imperative to include appropriate negative controls consisting of respective solvent, while screening natural products for their antimicrobial and/or anti-QS potential[38]. Lower concentrations of solvents, which apparently do not affect the bacterial growth and/or QS significantly, may still potentiate the effect of compound(s) under test.

Plant products which can promote growth of *C. violaceum* can be useful during such biological operations where this bacterium is used as process organism *e.g.*, reduction of chloride content from soil, biosorption of chromium, *etc*[28]. Further such QS-promoting plant products may be of possible use wherever increased communication among Gram-negative bacteria (which use a QS regulatory system identical to *C. violaceum*) is desired *e.g* in fermentations employing Gram-negative bacteria as process organism.

Plant products either inhibiting or enhancing QS-regulated violacein production in *C. violaceum* might exert their effect either by directly (positively or negatively) affecting the violacein synthesizing machinery or by interfering with the QS machinery. Two independent processes are involved in

biosynthesis of violacein in *C. violaceum*-the enzymatic process catalysed by five proteins VioABCDE, or the alternative non-enzymatic oxidative decarboxylation reactions[39]. Anti-QS potential of the natural products against Gram-negative bacteria may be owing to their ability to target the signal generator, the signal molecule, and the signal receptor of the QS system. Such products may prevent the signal molecules from being synthesized by the luxI-encoded AHL synthase. In absence of AHL, the bacteria will be rendered unable to sense when a quorum is reached, and will fail to activate the QS-controlled genes. The phytoconstituents of the active extracts may bind the QS signals making them unavailable for participating in QS-controlled metabolic processes. Alternatively such phytocompounds may target the LuxR signal receptor preventing binding of the signal molecules to the LuxR protein. This will not allow the LuxR protein to act as transcriptional regulator[3]. It is also possible that some of the components of natural extracts may be the analogs of bacterial AHL precursors, and may interrupt the AHL biosynthetic pathway[40]. Similarly the QS-enhancing products may promote synthesis of signal molecules and/or their binding with the LuxR protein. Garlic extract was shown to target 34% of the QS genes in *P. aeruginosa*, and RhlR was indicated to be the target of QS-inhibitor(s) present in garlic extract[41].

During all these experiments we used streptomycin (HiMedia) as a positive control. We thought it worthy to test with some other organism too, whether sub-MIC concentration of streptomycin can inhibit QS-regulated pigment production there too. We challenged *S. marcescens* with this antibiotic at 5 μg/mL, which resulted in a minor reduction in growth, but heavy reduction in production of prodigiosin. Latter is a red pigment whose production in *S. marcescens* is activated at high cell density by QS mechanisms[42]. Our findings are in contrast to those of Liu *et al*[43]. They reported antibiotics (amikacin, gentamycin, tetracycline, and erythromycin) at subinhibitory concentrations to improve the QS behaviour of *C. violaceum*. Antibiotics like vancomycin, tetracycline, ampicillin and azithromycin were reported to activate the expression of QS-related virulence factors in a QS-independent manner[44]. Ceftazidime and tobramycin were found to reduce C12-homoserine lactone and C4-homoserine lactone in *P. aeruginosa*[45]. Thus there may be some possible link between antibiotic signalling and QS. Antibiotics can also function as intermicrobial signalling molecules, and not only as a weapon during interspecies competition. However, different antibiotics may have different effects on QS pathways and QS-regulated physiological processes of bacteria.

This study has identified *P. pinnata* seed extracts and prodigiosin to possess anti-QS, and *M. hexandra* and *P. pyrifolia* seed extracts to possess QS-enhancing effect in *C. violaceum*. QS-enhancing extracts may find application for getting higher production of violacein, which is an attractive bioactive molecule. The anti-QS products can find applications in development of anti-pathogenic therapies, as an alternative to conventional antibiotic therapy which has an inherent obvious drawback of rapid development of drug resistance among the target pathogens[3]. QS-inhibiting compounds have considerable scope in therapeutics, and optimizing the use of conventional antibiotics. Nutraceutical preparations or functional foods for controlling immunocompromised individuals may be developed based on the natural products containing anti-QS ingredients[46]. Antiseptic ointments, eardrops, and dentifrice formulations with anti-QS ingredients can also be developed. Additionally, this study has shown QS-enhancing effect of DMSO, and QS-suppressing effect of methanol and ethanol, emphasizing the significance of inclusion of appropriate negative controls of these solvents in such experiments. Further investigation on the mode of action of such QS-promoting extracts can provide useful insights regarding the molecular basis of enhanced violacein production.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors thank Nirma Education and Research Foundation, Ahmedabad for financial and infrastructural support; Dr. Himanshu Pandya for authentication of plant materials; Dr. Mili Das and Palak Patel for access to HPLC; and Niral Sarvaiya for assisting in experiments with streptomycin.

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